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1 Basophil Activation Testing in Diagnosis and Monitoring of Allergic Disease – an overview

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39

40 Keywords

41 1. Basophil granulocyte

42 2. CD63

43 3. BAT, Basophil activation test

44 4. Allergy Diagnosis

45 5. Allergy monitoring

46 6. Allergen provocation /challenge testing

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Abstract

The nature of basophil activation as an ex vivo challenge makes it a multifaceted and promising tool for the allergist. Through development of flow cytometry, discovery of activation markers such as CD63 and markers identifying basophil granulocytes the basophil activation test (BAT) has become a pervasive test. BAT measures basophil response to allergen crosslinking IgE on between 150 and 2000 basophil granulocytes with remarkable analytical sensitivity in less than 0.1 ml fresh blood. Dichotomous activation is assessed as the fraction of reacting basophils. In patients with food-, insect venom-, and drug allergy as well as with chronic urticaria BAT can be part of the diagnostic evaluation in addition to history, skin prick testing and specific IgE determination. BAT may be also helpful in determining the clinically relevant allergen. Basophil sensitivity may be used to monitor patients on allergen immunotherapy, anti-IgE treatment or in the natural resolution of allergy. The test may use fewer resources and be more reproducible than oral, sting, nasal or bronchial challenge testing. BAT may be useful before challenge testing as it is less stressful for the patient and avoids severe allergic reactions. An important prospective step is to standardize BAT and make it available in diagnostic laboratories.

95 **Introduction**

96
97 The clinical impact of BAT is due to the unique ability of basophils to degranulate upon cross-
98 linking of the specific IgE (sIgE) bound on membrane-bound high affinity IgE-receptor (FcεRI) by
99 allergen exposure. After discovery of the quantal upregulation of CD63 during basophil activation
100 in 1991(1), the BAT was developed in the 90's (2). CD63 is a membrane protein localized to the
101 same secretory lysosomal granule that contains histamine. Translocation of CD63 to the cell
102 membrane during degranulation can be measured by flow cytometry. BAT (like SPT) reflects a
103 functional response as basophil (or skin mast cell) activation can be induced by cross-linking of
104 FcεRI.

105 In this overview, adapted from the EAACI Task force position paper (3) we provide an overview of
106 the practical and technical details as well as the utility of BAT in diagnosis and management of
107 allergic diseases.

108

109 *Blood samples for BAT*

110
111 Antihistamines do not interfere with BAT, but systemic steroids and cyclosporin A should be
112 avoided (4,5). It is recommended to take blood samples within one year of the most recent exposure
113 to the allergen source (6,7). Blood samples can be used within 24 hours (8), even though basophils
114 may lose reactivity. As there is diurnal variation in the reactivity to CD203c (9), timing of blood
115 sampling may be important. Tests done with whole blood are most commonly utilized, but
116 separation of cells from protective elements found in plasma may optimize activation through cell-
117 bound sIgE.

118 Interleukin-3 (IL-3) enhanced kinetics, reactance and sensitivity of blood basophils to FcεRI
119 mediated activation independently of extracellular Calcium. It enhances also the allergen specific
120 up-regulation of CD63 (4,10), but unspecifically upregulates CD203c (11).

121

122 *Selection of the source of allergen extracts (Box 1)*

123 Optimized concentrations for a wide range of allergens, allergen sources and allergen extracts
124 are listed in the original position paper in table S1 (3). Furthermore, optimized allergen
125 preparations are available from vendors. Drug allergens are typically active in the mg/ml range,
126 and can be diluted 5- to 25-fold. Pure active ingredients or injectable intravenous drug preparations
127 should be used when possible since solubilized tablets are complex mixtures of drugs and
128 excipients.

129 Protein allergens are often used in concentrations starting in the µg/mL range, and may be diluted
130 up to 5 – 15 log concentrations to ng/ml - pg/ml before reactivity is lost. If recombinant allergen
131 preparation or purified allergens are used for BAT, the molar concentration of allergens enables
132 very precise analyses.

133 This standardized allergen preparation is essential when comparing basophil sensitivity data.

134 *Flow Cytometry in BAT*

135
136 At the moment BAT with CD63 is the best clinically validated test (12,13,14), but the BAT
137 based on CD203c has been shown to be a reliable test (15,16).

138 Basophils can be identified with different combinations of antibodies in flow cytometry. They
139 were first identified as circulating IgE⁺ cells. However, low side scatter in combination with
140 CD123⁺/HLADR⁻, CRTH2⁺, CD203c⁺ or CD193⁺ are commonly applied combinations. Cell
141 surface expression of the basophil selection marker CD193 (CCR3) was more stable than IgE
142 or CD123/HLA DR on resting basophils (17). IgE and CD123/HLA-DR showed somewhat
143 more inter-individual variability in cell surface expression. CD203c can be used for both
144 identification and as an activation marker. Quality of blood basophils obtained is usually
145 confirmed by stimulation with the bacterial peptide fMLP. Anti-IgE or anti-FcεRI antibodies
146 are used as IgE-mediated positive controls, buffer as negative control.

147 If standardized commercial tests are not used, the method used for testing has to undergo
148 validation.

149

150 *Presentation and interpretation of BAT*

151 There are two common measures of basophil activity; basophil reactivity (2), the number of

basophils that respond to a given stimulus, and basophil sensitivity (1, 18), the allergen concentration at which half of all reactive basophils respond (Fig. 1A). Basophil reactivity depends on the priming state of the basophil and the cellular translation of the IgE signal within the cell (19). It is sufficient to measure reactivity at one or two concentrations, and assessment of basophil reactivity is important using a positive control before basophil sensitivity to allergen is measured.

Basophil sensitivity is a function of reactivity and the compound affinity of cell-bound sIgE for allergen and free competing immunoglobulin. It requires measurement of reactivity at 6-8 allergen concentrations. The graded response to allergen is fitted to a curve of reactivity versus allergen concentration, and the eliciting concentration at which 50% of basophils respond (EC50) is determined (Fig. 1 B). EC50 can be expressed as 'CD-sens' by inversion and multiplication by 100 (20).

More recently the area-under-the-dose curve (AUC) measurement attempts to combine reactivity and sensitivity into one (Fig. 1C); it is similar to a coordinate system of sensitivity and reactivity, but also incorporates partial anergy induced at high allergen concentrations and can be calculated even in cases where responses do not fit well to a typical dose-response curve (21). ROC curves are used in identification of novel allergens when ≥ 7 sensitized patients are available.

Basophil granulocytes of non-responders (6%-17% of population) can remain unresponsive to stimulation through Fc ϵ RI. It is attributed to differences in the intracellular signaling pathway. Results from non-responder patients should be regarded as false negatives.

Placing BAT in the diagnostic algorithm for allergic disease

In the general algorithm for diagnosis of allergy (Box 2), patient history should be taken with an attempt to identify the allergen source and assess the severity of the allergic reaction. The allergic response should be confirmed by measurement of sIgE, skin prick testing and, for insect venom and drug allergy, intradermal testing. Measurement of sIgE may not be possible if the allergen source is not available as a routine reagent, and may be of limited value depending on the performance of the available reagents. BAT is a functional test resembling

181 an *ex vivo* IgE-mediated cellular response . It can be measured at the same time as sIgE, and in
 182 general precedes *in vivo* provocation tests.

183

184 **Chronic Urticaria**

185 The mechanism of chronic spontaneous urticaria (CU) is still incompletely understood. About half
 186 of the patients have autoantibodies against FcεRI and a few against IgE. CU sera activate resting
 187 basophils of normal donors to upregulate CD63 and CD203c. BAT may replace the autologous
 188 serum skin test (ASST) (22).

189 BAT with CD63 upregulation as an activation marker for CU was established as a specific,
 190 sensitive and safe *in vitro* alternative to detect functional autoantibodies (10, 23, 24). The central
 191 problem is the heterogeneity of the results using different basophil donors. This can be normalized
 192 by titrated addition of IL-3 (10). BAT with autologous basophils should not be performed because
 193 CU patients are often non responders or poor responders to IgE crosslinking and have diagnostic
 194 basopenia.

195 *Key messages*

- 196 • BAT may replace ASST as the standard diagnostic procedure to identify autoreactive serum
- 197 factors in CU with a quantifiable result that may be used to monitor treatment.
- 198 • BAT removes the risk of accidental infection.
- 199 • In contrast to ASST, there is no need to suspend antihistamines, as they do not influence the
- 200 result of BAT.

201

202 **Drug Allergy**

203 The diagnostic work-up of drug hypersensitivity reactions (DHR) aims to identify the culprit agent,
 204 identify cross-reactive drugs and to determine a safe alternative drug (25). Here BAT is an
 205 additional tool that is safer, gentler and cheaper than a challenge and, in some instances, is the only
 206 available diagnostic tool. The sensitivity of BAT in diagnosis of drug allergy is about 50%, and the
 207 specificity up to 93%.

208 There are several studies including BAT in drug allergy diagnosis for beta-lactams, NMBA,
 209 quinolones, radio contrast media and pyrazolones with good sensitivity and specificity.

BAT provides positive results in 40% of the patients with immediate-type systemic reaction and negative skin test and confirmed by provocation that constitute about 25% of all beta-lactam-allergic patients. BAT has also a good negative predictive value, useful in the decision to perform the provocation test as demonstrated with quinolones. Furthermore, it has a complementary role to skin tests for different drug hypersensitivities and can be particularly useful in the study of cross-reactivity between NMBA, for the identification of safe alternatives for future surgery (25, 26).

Key Messages

- For a number of drugs, BAT is the only available test to confirm a hypersensitivity response.
- A negative test does not rule out that the patient reacts to a metabolite of the drug.
- It may be difficult to confirm the clinical history of hypersensitivity by BAT after 18 months from the most recent clinical reaction.
- Once the hypersensitivity is established, cross-reacting drugs and safe alternatives may be suggested by BAT.

Food allergy

The performance of BAT in the diagnosis of food allergies has been assessed in various studies. The reported sensitivity of BAT ranges from 77-98%, and the specificity from 75-100%. BAT in these studies was more accurate than sSPT and sIgE (27, 28). For single individuals BAT sensitivity seems to allow a risk estimation for severe clinical reactions: In peanut allergy, BAT significantly improved clinical diagnosis over the use of SPT and sIgE and reduced the number of OFC required. BAT showed 100% specificity, suggesting that in patients with a positive BAT the OFC could be deferred (29).

Patients with clinical allergy that developed symptoms in an OFC to peanut had high basophil sensitivity to peanut, and patients who tolerated peanuts in a OFC had low basophil sensitivity to peanut. Although OFC and basophil sensitivity both identified all clinically sensitized children, only basophil sensitivity was reproducible at two consecutive visits ($r^2=0.94$). In a recent publication, BAT reactivity reflected the allergy severity and BAT sensitivity reflected the threshold of response to the allergen source in an OFC (30).

240

241 It has been shown that basophil reactivity distinguish patients that tolerate extensively heated forms
242 of cow's milk and egg from patients who do not. BAT may be useful in assessing the natural
243 resolution of food allergies that are commonly outgrown over time, such as cow's milk allergy (28),
244 and in determining when the food can safely be reintroduced in the diet. BAT has also been used to
245 monitor clinical response to immune-modulatory treatment of food allergy.

246 Basophil CD203c expression has shown to decrease during treatment with Omalizumab and to
247 return to pre-treatment levels after cessation of therapy in patients with peanut allergy (31). Also
248 improvement in basophil sensitivity to milk of milk allergic children treated with Omalizumab
249 predicted tolerance in a milk challenge test.

250

251

252 *Key messages*

- 253 • BAT can improve the diagnosis of food allergy in addition to SPT and sIgE and may be
254 able to reduce the number of OFC.
- 255 • BAT can be used to monitor the natural resolution and clinical response to immune-
256 modulatory treatments for food allergy.

257

258 **Hymenoptera venom allergy**

259 Overall, the diagnostic sensitivity of BAT with insect venoms referred to the history was found to
 260 be 85%-100%, the diagnostic specificity 83%-100% (13,14,15). Specific diagnostic problems can
 261 be resolved by measuring basophil reactivity and sensitivity.

262 *BAT in patients with negative standard tests:* A subset of patients (4-6%) with a history of systemic
 263 reactions after Hymenoptera stings have negative venom-specific IgE and skin test results. BAT
 264 allows the identification of about two thirds of those patients (32).

265 *BAT in patients sensitized to bee and wasp venom “double positivity”:* Up to 60% of the patients
 266 with Hymenoptera venom allergy have sIgE to both bee and wasp venom. Basophil reactivity has
 267 the lowest rate of double positivity of diagnostic tests for hymenoptera allergy (33) and repeatedly
 268 shows a positive result to only one venom in about one-quarter to one-third of patients with double
 269 sIgE positivity (13 34). In the case of patients with double positive BAT, the venom to which the
 270 patient is markedly more sensitive might represent the primary sensitizing allergen source, but this
 271 requires further research. BAT adds more clinically relevant information about the culprit insect
 272 than component-resolved sIgE testing with single recombinant allergens (32, 34). However,
 273 recombinant venom allergens applied to BAT might represent a step forward in developing better in
 274 vitro tests for specific diagnosis of Hymenoptera allergy.

275 *Monitoring the effect of venom immunotherapy with basophil sensitivity:* A clear decrease in
 276 basophil sensitivity is found up to 4 years after initiation of VIT, without a change in basophil
 277 reactivity. A recent report about an 8-year follow up of patients submitted to VIT showed that the
 278 decrease in basophil sensitivity seemed to be also associated with the induction of tolerance (35).
 279 Some studies suggest that side effects during the build-up phase of VIT are predicted by a high
 280 basophil sensitivity (36).

281 *Key messages*

- 282 • Basophil reactivity and sensitivity (in that order) play an important role in the diagnosis of
- 283 venom allergy, as they are effective tools to identify the primary sensitizing antigen.
- 284 • The utility of basophil sensitivity as the tool of choice to monitor the effect of VIT should be
- 285 explored.

286

287 **Inhalant allergens**

288 Determination of sIgE or skin testing in combination with the clinical history are usually sufficient
289 to diagnose allergy to inhalant allergens. However, in specific cases BAT can be helpful for
290 diagnosis. Patients with local allergic rhinitis by nasal provocation who have no detectable sIgE or
291 skin testing but have a positive BAT are a notable example (37). Crude allergen sources as well as
292 modified and recombinant allergens have been tested with good outcomes, Basophil sensitivity
293 correlates with the nasal provocation titer in allergic rhinitis (38), the allergen specific bronchial
294 provocation threshold in allergic asthma and the asthma control test. This indicates that basophil
295 allergen threshold sensitivity (CD-sens or EC50) may accurately reflect clinical allergen sensitivity
296 (39).

297 *Monitoring the effect of allergen immunotherapy and anti-IgE treatment effect:* Basophil
298 sensitivity can be used to assess the efficacy of allergen-specific immunotherapy (AIT) to
299 aeroallergens. It has been used to monitor patients treated with AIT for birch and timothy (18,19),
300 and showed reduced allergen sensitivity already during the up-dosing stage. Basophil sensitivity has
301 also successfully been used to identify patients who respond to the humanized monoclonal anti-IgE
302 antibody Omalizumab and to assess treatment efficacy (40).

303 *Key message*

- 304 • Basophil sensitivity has the unique ability to monitor a patient's inhalant allergen sensitivity
305 over time, to measure natural progression of allergy, and may be developed to serve as a
306 tool to measure the response to treatment with AIT and Omalizumab.

307 308 *Perspectives*

309 Different methods of reporting results of BAT may be useful when asking different clinical
310 questions; stimulation index and % positive basophils are used. When reactivity is measured in
311 clinical settings, the aim is usually to identify an allergen concentration at which change in
312 sensitivity is optimally identified. Basophil sensitivity is used to monitor change in allergic disease.
313 Both reactivity and allergen sensitivity are measured when allergy severity is evaluated by basophil
314 sensitivity, but a useful composite measure has yet to be designed.

315 A threshold for basophil reactivity is often set at 2%, 10% or 15% of resting basophils. An
316 alternative method would be to set the threshold halfway between the MFI of resting basophils and
317 the positive control.

318 Major applications of BAT are summarized in Box 4. Laboratory procedures and allergen
319 concentrations in BAT should be standardized e.g by the use of industry standards like MiFlowCyt
320 or purchase of standardized material from CE-approved vendors. An important next step is the
321 standardization and automation of analysis of BAT. Then it will be possible to perform large
322 multicenter trials to characterize the diagnostic performance of BAT and broaden its use as a
323 clinical tool. Such studies should also address the relationship of measures of BAT and sensitivity
324 to sIgE, clinical symptoms and symptom severity.

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514 **Figure 1: Assessing basophil response.**

515 The fraction of CD63⁺ basophils is plotted against log allergen concentration. Adapted from
516 (20) with permission from the authors.

517 A. **Basophil reactivity** is the dose (range) at which maximal response occurs. **Basophil**
518 **sensitivity** is the dose at which half of the maximal response occurs. *At high allergen
519 concentrations, basophil response may be suppressed.

520 B. A change in sensitivity toward higher allergen concentration is the most reproducible
521 basophil biomarker for reduced clinical sensitivity to allergen to date. Attempts to reduce the
522 number of BAT tests required to determine a significant change in basophil response have
523 focussed on identifying an allergen concentration at which a change in sensitivity can readily
524 be assessed (grey box).

525 C. Basophil response could also be assessed as area under the curve (AUC) with a log allergen
526 axis, or a similar composite measure reflecting both reactivity and sensitivity. Variation in
527 maximal basophil reactivity arises concurrently with, and may be inseparable from, a change
528 in sensitivity.

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544 **Conflict of interest**

545 AFS, BE, SUP, WGS, OVH obtained research funding, and BE and OVH received speaker honoraria from Bühlmann
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548 that there are no conflicts of interest.